CHAPTER 3
MATERIALS

I. Chemicals
List of chemicals procured from different companies

A. Qualigens, India
Agar, Beef extract, Casein, Crystal violet, Dipotassium hydrogen orthophosphate, Ferrous sulphate, Glucose, Glycine, Glutaraldehyde, Grams iodine, Hydrogen peroxide, Hydrochloric acid, Iodine, Lactose, Maltose, Methanol, Potassium nitrate, Phenol red, Peptone, Potassium dihydrogen orthophosphate, Sodium bicarbonate, Safranin, Sucrose, Sodium chloride, Sodium citrate, Sodium thiosulfate, Starch, Sodium alginate, Sodium potassium tartrate, Trichloroacetic acid, Urea, Yeast extract.

B. Himedia, India
Agar agar, Acetic acid, Ammonium persulphate, Bovine serum albumin, Collagen, Egg albumin, Ethylenediaminetetraacetic acid (E.D.T.A.), Gelatin, Sodium alginate, α, N, N', N'-tetramethylethlenediamine (TEMED), Tris (hydroxyl methyl) amino methane hydrochloride (Tris-HCl).

C. Sigma-Aldrich, USA
Acrylamide, Bisacrylamide, Bromophenol blue, Coommassie brilliant blue R-250, Diisopropyl fluorophosphate (DFP), DEAE-Cellulose, p-Chloromercuric benzoate, Phenylmethysulfonylfluoride, β-mercaptoethanol, Sephadex-G-100, Iodoacetate.
II. Solutions

Solutions of solids were prepared on (w/v) basis and of liquids (v/v) basis, unless otherwise stated.

III. Experiments: All experiments were carried out in triplicate.
3.1 COLLECTION OF SOIL SAMPLES.

Soil samples were collected in sterile bottles from different locations, where effluents are being discharged by M/s Nellore Detergent industry located at Nellore, Andhra Pradesh. Soil samples were also collected from locations very adjacent to M/s Nellore Detergent Industry and used as control. Soil samples were air-dried and mixed thoroughly to increase the homogeneity and sieved through < 2 mm mesh. These soil samples (1g) were diluted with water (100ml) and incubated in boiling water bath for enough time (30-60 min) to kill most microorganisms except temperature resistant microorganisms and spore formers as described by Lichstein et al. (1944).

3.2 ENUMERATION OF MICROFLORA IN SOIL SAMPLES

3.2.1 Enumeration of bacterial populations

Bacterial populations in soil suspension with/without effluent discharges were enumerated on Nutrient agar medium with the following composition.

- Peptone : 5.0g
- Beef extract : 3.0g
- Agar agar : 20.0g
- Distilled water : 1000ml
- pH : 7.2
Uniform soil suspension was prepared by adding one gram of soil sample with/without effluent discharges to 10ml of sterile distilled water separately and tubes were shaken well for homogeneity. From this soil suspension, serial dilutions were made up to $10^{-5}$. From each dilution, 0.1ml aliquots of soil suspension were placed on the surface of nutrient agar medium and spread with the help of glass spreader under aseptic conditions. These plates were incubated for 48 h in an incubator at 37$^\circ$C. Bacterial colonies developed on plates were counted by Qubic colony counter.

### 3.2.2 Screening of bacterial isolates for alkaline protease production

Bacterial colonies appeared in plates for enumeration of bacterial flora in soil suspension of both control and effluent discharged soils were further purified by subculturing number of times in Yeast extract casein medium (YEM) with the following composition.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0g</td>
</tr>
<tr>
<td>Casein</td>
<td>0.5g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.1g</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.2g</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>1.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>
The Bacterial isolates were finally maintained on the same YEM slants. The enriched purified bacterial isolates were screened for protease production by casein hydrolysis method. Casein hydrolysis was performed by supplementing the nutrient agar medium with skim milk (1%, w/v) with the following composition.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td>100g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

The above ingredients were dissolved, pH of the medium was adjusted to 7.2 and sterilized by autoclaving at 15lb pressure for 15 min. Autoclaved medium was allowed to cool (45°C-50°C) and poured into sterile petri plates (20ml each) and allowed to solidify. Single line streak inoculation of bacterial isolate was made on surface of the medium along with the control. Plates were incubated for 24 to 48 h at 37°C in an inverted position. The plates were observed for clearing zone around the line of growth.

The bacterial cultures were examined for various morphological and biochemical characteristics as per Bergeys Manual of Determinative Bacteriology (Holt et al., 1994).
3.2.3 Protease activity of bacterial cultures isolated from soil samples

3.2.3.1 Determination of bacterial growth

Bacterial culture was grown in the manner as described by Tsujibo et al. (1990) for determination of bacterial growth and production of proteolytic enzymes. Bacterial culture was cultivated in YEM with the following ingredients in g/L: glucose, 1.0; casein, 0.5; yeast extract, 0.5; KH$_2$PO$_4$, 0.1; MgSO$_4$, 0.2 and Na$_2$CO$_3$, 10. The pH of the medium was adjusted to 7.2. The medium was inoculated with 5ml of broth culture in lag phase and the flasks were incubated at 37°C for 120 h on a rotary shaker. Optical density (OD) of the broth was measured at 650nm for every 6 h interval of time by taking 1ml of broth culture.

3.2.3.2 Estimation of protein content

Protein content of the crude sample was estimated by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. 20mg of BSA was dissolved in 100ml of 0.9% NaCl and the solution was pipetted into series of test tubes (containing 40 to 200μmol). 1ml of double distilled water was added to give final volume of 1ml. 5ml of alkaline copper reagent was added, mixed and allowed to stand at room temperature for 10min. 0.5ml of Folin-Ciocalteau reagent was added and mixed well immediately. After 30min of incubation at room temperature, OD was read at 660nm in ELICO UV-visible spectrophotometer (Model SL-164).

3.2.3.3 Assay of protease activity by casein digestion method

The proteolytic activity of culture filtrate was assayed by casein digestion method (Manachini et al., 1988). At the end of fermentation period, the culture medium was centrifuged at 10,000 rpm for 15 min to obtain the crude extract, which was used as an enzyme source.
The reaction mixture was prepared by adding 0.5ml Tris-HCl buffer (50mM, pH 8) and 1 ml of 1% casein in Tris-HCl buffer of same pH to 0.5ml of culture filtrate. Boiled enzyme filtrate served as control. The mixture was incubated for 30 min at 45°C and the reaction was stopped by the addition of 4ml of 5% Trichloroacetic acid (TCA). After 60 min the solution was filtered through Whatman No.1 filter paper and 1ml of filtrate was mixed with 5ml of 0.4M Na₂CO₃ followed by the addition of 0.5ml phenol reagent (1:1 dilution with distilled water). The liberation of tyrosine (appearance of blue colour) was determined at 660nm in a spectrophotometer (ELICO, model SL-164). One unit of protease activity is defined as the amount of which liberates 1μmol of tyrosine ml⁻¹ min⁻¹ under experimental conditions.

3.3 Optimisation of protease production by *Bacillus subtilis* K-30

3.3.1 Determination of effect of rice bran on protease production by *B. subtilis* K-30 in three different media

Sterile fifty milliliters of three different media-minimal, YEM, and glucose gelatin broths were separately distributed into sterile 250ml Erlenmeyer flasks. Ingredients of three media are mentioned below. All three media were amended with rice bran at 0.5% to 3% level. Meanwhile, growth suspension culture in sterile medium was prepared from 48 h old culture of *B. subtilis* K-30 grown in YEM.
Glucose yeast extract medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0g</td>
</tr>
<tr>
<td>Casein</td>
<td>0.5g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.1g</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.2g</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>1.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
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</tr>
</tbody>
</table>

Glucose gelatin medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient gelatin</td>
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<tr>
<td>K$_2$HPO$_4$</td>
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<tr>
<td>CaCl$_2$</td>
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</tr>
<tr>
<td>Distilled water</td>
<td>100ml</td>
</tr>
<tr>
<td>pH</td>
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</tbody>
</table>

Minimal medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO$_3$</td>
<td>2.0g</td>
</tr>
<tr>
<td>KCl</td>
<td>2.0g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.0g</td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>1.0g</td>
</tr>
<tr>
<td>(NH$_4$)H$_2$PO$_4$</td>
<td>1.0g</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.5g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.9</td>
</tr>
</tbody>
</table>
3.3.2 Determination of effect of inoculum size on protease production by *B. subtilis* K-30

To study the effect of inoculum size on protease production, 50ml YEM amended with 1% rice bran was distributed into each 250ml Erlenmeyer flask. Five milliliter of sterile distilled water was aseptically placed on surface of yeast extract casein agar slant containing 6 day old culture of *B. subtilis* K-30 and a suspension was prepared to give final concentration. The flasks were inoculated with desired sizes of inoculum i.e. in the range of 0.2%-1.4% and incubated for growth as described in the section 3.2.3.1. At the end of 48 h incubation, the flasks were processed for determination of protease activity.

3.3.3 Determination of effect of pH of the medium on protease production by *B. subtilis* K-30

In view of maximum growth and high protease production on yeast extract casein medium supplemented with 1% rice bran, further experiments were carried out in the same medium at different pH values to study the effect of pH on protease production. Fifty milliliters of YEM amended with 1% rice bran were dispensed into 250 ml Erlenmeyer flasks and the pH was adjusted in the range of 4-12. The flasks were inoculated with growth suspension of *B. subtilis* K-30 and grown in axenic conditions in the same manner as specified above (section 3.2.3.1). The enzyme activity of protease in the culture filtrate of *B. subtilis* K-30 after 48 h of its growth in the medium were determined as specified in section 3.2.3.3.
3.3.4 Determination of effect of temperature on protease production by 
*B. subtilis* K-30

In view of maximum growth and high protease production on YEM supplemented with 1% rice bran further experiments were carried out at different temperatures (30-90°C) to study the effect of temperature on protease production. Fifty milliliters of yeast extract casein medium amended with 1% rice bran adjusted to pH 9 were dispensed into 250ml Erlenmeyer flasks. The flasks were inoculated with growth suspension of *B. subtilis* K-30 and grown in axenic conditions, maintained at different temperatures i.e. 30-90°C. The enzyme activity of protease in the culture filtrates of *B. subtilis* K-30 after 48 h of its growth in the media were determined as specified in section 3.2.3.3.

3.3.6. Determination of effect of supplementation of different carbon sources on protease production by *B. subtilis* K-30

In order to determine the effect of supplementation of different carbon sources on protease production by *B. subtilis* K-30, different carbon sources such as glucose, skim milk, lactose, sucrose, starch, wheat bran and rice bran were added at 1%(w/v) level to 50 ml of yeast extract medium in 250ml Erlenmeyer flasks. The flask with casein amended medium devoid of carbon source, served as control. After sterilization, the flasks were aseptically inoculated with bacterial suspension and grown in axenic conditions as detailed in section 3.2.3.1. All flasks growing in standard conditions were withdrawn after 48 h and activity of the enzyme in the culture filtrates were determined as described in section 3.2.3.3.
3.3.7. Determination of effect of supplementation of different nitrogen sources on protease production by *B. subtilis* K-30

Yeast extract medium amended with 1% rice bran was dispensed to 250ml Erlenmeyer flasks at the rate of 50ml medium per flask. Only one nitrogen source gelatin or peptone or soybean meal or glycine or tryptone or beef extract at a concentration of 1% (w/v) was added to each flask. The flask with casein amended medium devoid of nitrogen source, served as control. All flasks were aseptically inoculated with bacterial suspension of 24 h old *B. subtilis* K-30 and incubated for growth as specified in section 3.2.3.1. The flasks were processed at the end of 48 h incubation for measurement of activity of protease enzyme in the culture filtrate in the manner specified in section 3.2.3.3.

3.4 Purification and characterization of thermostable alkaline protease of *B. subtilis* K-30

3.4.1 Culturing of *B. subtilis* K-30 for optimal production of thermostable alkaline protease

*B. subtilis* K-30 was cultured on YEM supplemented with appropriate nutrients such as rice bran at 1% (w/v), inoculum size at 1% (v/v) and pH 9 was maintained. Sterile 1000ml Erlenmeyer flasks, containing sterile 250ml of the medium, were inoculated with 48 h old culture of *B. subtilis* K-30. The flasks were incubated for growth at 55°C for 96 h on an orbital shaker with 150 rpm and the culture filtrate was harvested as described earlier in section 3.2.3.3. The culture filtrate served as crude fraction and was used as source of alkaline protease. The protease activity of culture filtrate was determined by casein digestion method by collecting the culture filtrate at an interval of 12 h upto 96 h.
3.4.2 Purification of thermostable alkaline protease of *B. subtilis* K-30

*B. subtilis* K-30 was cultured in 2000ml sterile Erlenmeyer flask containing 500ml yeast extract casein medium amended with rice bran at 1% (w/v), inoculum size at 1% (v/v), and maintained at pH 9. The organism was allowed to grow for 48 h as described in section 3.4.1. The cells were separated by centrifugation and the supernatant was fractionated by ammonium sulphate.

3.4.2.1 Ammonium sulphate precipitation

The culture filtrate obtained was used for purification of protease and all steps of purification were carried out at 4°C. The protein from culture filtrate of *B. subtilis* K-30 was precipitated with solid ammonium sulphate (60% w/v) and kept overnight at 4°C. The resulting precipitate was collected by centrifugation at 10,000g for 30 min at 4°C in a high speed cooling centrifuge (Shimadzu). The precipitate was dissolved in 0.1M Tris-HCl buffer, pH 7.8 and dialyzed in a dialysis bag (Sigma, USA) overnight against the same buffer. During the course of dialysis the buffer was frequently changed with the fresh lot until no traces of ammonium were found in the buffer upon testing with Nesslers reagent. The dialyzed fraction was considered as ammonium sulphate precipitated fraction. Aliquots of this fraction were used for determining the activity of protease and protein content as described in sections 3.2.3.3 and 3.2.3.2 respectively.

3.4.2.2 DEAE-Cellulose Chromatography

DEAE-Cellulose was suspended in water for one day at room temperature and then washed thoroughly 2-3 times with water. The suspension was then packed in a column (2 X 35cm) and DEAE-Cellulose column was activated by repeated washings with a solution prepared by adding 50mM Tris-HCl buffer, pH 8, 100mM EDTA and
50mM NaCl. The packed column was equilibrated with 20mM Tris-HCl buffer, pH 8.0 and 20mM CaCl₂. The enzyme from the preceding step was loaded on DEAE-Cellulose column. The equilibrating buffer was used for preliminary washing of unbound proteins until the eluent was free from proteins. The protein elution pattern was monitored spectrophotometrically at 280nm. The bound proteins were eluted with sodium chloride of ionic strength, 0.5M in equilibrating buffer at a flow rate of 40ml/h. Fractions of 5ml were collected and analysed for protein content and protease activity. Fractions 16-28 showing higher enzymatic activity were pooled. The proteins from the pooled fractions were brought to 60% saturation with pulverized ammonium sulphate at 4°C. The collected pellet was dissolved in minimal volume of 0.1M Tris-HCl buffer, pH 7.8 and dialyzed in a dialysis bag against the same buffer.

3.4.2.3 Gel filtration Chromatography

The dialysate obtained from DEAE-Cellulose chromatography was subjected to gel filtration on Sephadex G-100 column. Three grams of Sephadex G-100 was suspended in distilled water and kept overnight for swelling with intermittent stirring at shorter intervals to prevent the formation of lumps. The swelling gel bead solution was poured into column tube (1.5 X 24 cm), which was previously inserted with glass wool at the bottom. The gel beads were allowed to settle gently without trapping of air bubbles. The prepared column was pre-equilibrated with 0.1M Tris-HCl buffer, pH 7.8. The dialyzed and concentrated fraction from DEAE-Cellulose chromatography was loaded on Sephadex G-100 column which is pre equilibrated with 0.1M Tris-HCl buffer; pH 7.8. The loaded column was eluted with the same buffer. One milliliter fractions of eluate were collected in clean dry test tubes in an automated fraction collector (Biorad, Model-2110) at 4°C and absorbance of one milliliter fractions at
280nm were measured in a UV-Visible spectrophotometer (ELICO-SL164) for protein content. Proteins in the sample (ammonium sulphate precipitated fraction) were resolved into peaks on the column. One milliliter fractions were pooled together and aliquots from pooled fractions were used for determining the activity of alkaline protease and protein content by using methods as specified in sections 3.2.3.3 and 3.2.3.2 respectively.

3.4.3 Characterization of thermostable alkaline protease of B. subtilis K-30

3.4.3.1 Determination of molecular weight of alkaline protease of B. subtilis K-30 by SDS-PAGE.

SDS-PAGE is frequently applied method for the determination of purity of the enzyme. SDS-PAGE was carried out as described by Laemmili (1970). The enzyme preparation was denatured by boiling in the presence of 1% SDS and 1% 2-mercaptoethanol and subjected to SDS-PAGE on slab gel with 1% stacking gel (pH 6.8) overlaid on 12% separating gel (pH 8.8). The separating gel consisted of 10% (w/v) acrylamide, N, N-methylene-bis-acrylamide at a concentration such that the ratio of monomers to bis was 30:08; 0.375M Tris-HCl (pH 8.8) and 0.1% sodium dodecyl sulphate. It was chemically polymerized with 0.05%(w/v) ammonium persulphate and 0.05%(v/v) TEMED. The solution was cast into slabs and was over layered with butanol to exclude contact with water. The stacking gel containing 4%(w/v) acrylamide, 0.12M Tris-HCl (pH 6.8), 0.1% SDS, 0.05%(w/v) ammonium persulphate, 0.05%(v/v) TEMED was over layered on separating gel. Samples having concentration range of 50-200µg were mixed with an equal volume of sample buffer having 0.0625M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.002% bromophenol blue and heated in a boiling water bath for 5 min. After cooling samples were loaded into the wells. The samples, purified protease and
protein standard markers were electrophoresed at 50V in stacking gel and 100V in resolving gel for about 6 h using 0.025M Tris, 0.192M glycine buffer (pH 8.3) containing 0.1% SDS as the electrode buffer. The protein bands separated on acrylamide gel were visualized by staining with coomassie brilliant blue R-250, 0.25% in 40% methanol and 7% acetic acid, followed by destaining in the solution containing methanol: acetic acid: water (40:5:55) (v/v). The molecular weight of purified protease was calculated on the basis of relative mobilities of protein standard markers.

3.4.3.2 Determination of effect of pH on protease activity in presence and absence of stabilizing agent

In order to determine the effect of pH on protease activity, the activity of crude and purified protease was measured at different pH values in the presence and absence of 10mM CaCl_2. Fifty milliliters of yeast extract casein medium amended with 1%(w/v) rice bran were dispensed into sterile 250ml Erlenmeyer flasks and pH was adjusted in the range of 5-12. The medium is inoculated with 48 h old culture of B. subtilis K-30 at 1%(v/v) inoculum size and kept on shaker at 150 rpm at 30°C for 48 h. After fermentation period, the culture medium was centrifuged at 10,000 rpm for 15 min to obtain crude extract which is used as an enzyme source. The reaction mixture (test) was prepared by adding 0.5ml Tris-HCl buffer (50mM, pH 8) and 1ml of 1% casein in Tris-HCl buffer of same pH to 0.5ml of culture filtrate. Boiled enzyme filtrate served as control. To study the stabilizing effect of CaCl_2 on crude enzyme, the above reaction mixture (test) is supplemented with 1ml of 10mM CaCl_2 and incubated for 5 min before the addition of culture filtrate. The mixture was incubated for 30 min at 45°C and the reaction was stopped by the addition of 4ml of 5% TCA. After 60 min the solution was filtered through Whatman No.1 filter paper.
and 1ml of filtrate was mixed with 5ml of 0.4M Na₂CO₃ followed by the addition of 0.5ml phenol reagent (1:1 dilution with distilled water). The liberation of tyrosine (appearance of blue colour) was determined at 660nm in a spectrophotometer (ELICO, model SL-164).

To study the effect of CaCl₂ on purified protease, the pH range 5-12 was maintained by using the following buffers (0.05M): phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0) and glycine-NaOH (pH 9.0-12.0). The purified enzyme was diluted to two fold dilution in different buffers (pH 5.0-12.0). The reaction mixture (test) was prepared by adding 0.5ml Tris-HCl buffer (50mM, pH 8) and 1ml of 1% casein in Tris-HCl buffer of same pH to 0.5ml of purified protease. Reaction mixture without purified protease serves as control. To study the stabilizing effect of CaCl₂ on purified protease, the above reaction mixture (test) is supplemented with 1ml of 10mM CaCl₂ and incubated for 5 min before the addition of purified protease. The reaction mixture was incubated at 45°C for 30 minutes, and the activity of enzyme was measured as described in the section 3.2.3.3.

For stability studies, the purified enzyme was diluted to two fold dilutions in different buffers (pH 5.0-12.0) and incubated at 45°C in presence of CaCl₂ for 2 and 20 h before the addition of substrate. The relative activity at each exposure was calculated using formula

\[
\% \text{Relative activity} = \frac{\text{Activity (Test)}}{\text{Activity (Control)}} \times 100
\]

One unit of protease activity is defined as the amount of which liberates 1μmol of tyrosine ml⁻¹ min⁻¹ under experimental conditions.
3.4.3.3 Determination of effect of temperature on protease activity in presence and absence of stabilizing agent

To determine the effect of temperature on protease activity, the activity of crude and purified protease was measured at different temperatures (30-90°C) in the presence and absence of 10mM CaCl$_2$. Fifty milliliters of yeast extract casein medium amended with 1%(w/v) rice bran were dispensed to sterile 250ml Erlenmeyer flasks and pH was adjusted to 9.0. The medium is inoculated with 48 h old culture of B. subtilis K-30 at 1%(v/v) inoculum size and kept on shaker at 150 rpm and maintained at different temperatures (30-90°C) for 48 h. After fermentation period, the culture medium is centrifuged at 10,000 rpm for 15 min to obtain crude extract which is used as an enzyme source. The reaction mixture (test) was prepared by adding 0.5ml Tris-HCl buffer (50mM, pH 8) and 1ml of 1% casein in Tris-HCl buffer of same pH to 0.5ml of culture filtrate. Boiled enzyme filtrate served as control. The activity of the crude enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30°C to 90°C in the presence and absence of 10mM CaCl$_2$ for 30 min. The reaction was stopped by the addition of 4ml of 5% TCA. After 60 min the solution was filtered through Whatman No.1 filter paper and 1ml of filtrate was mixed with 5ml of 0.4M Na$_2$C$_3$O$_3$ followed by the addition of 0.5ml phenol reagent (1:1 dilution with distilled water). The liberation of tyrosine (appearance of blue colour) was determined at 660nm in a spectrophotometer (ELICO, model SL-164).

The activity of the purified enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30°C to 90°C in the presence and absence of 10mM CaCl$_2$ for 30 min, relative protease activities were assayed as described in the section 3.2.3.3.
For thermostability studies, the reaction mixture was prepared by adding 0.5 ml Tris-HCl buffer (50 mM, pH 8) and incubated at different temperatures (60°C, 70°C and 80°C) for different periods of time (50-400 min) in presence of 1 ml of 10 mM CaCl₂. Relative protease activities were assayed as described in the section 3.2.3.3.

3.4.3.4. Determination of effect of protease inhibitors and chelators on protease activity

The effect of various inhibitors such as phenyl methyl sulfonyl fluoride (PMSF) and DFP (serine inhibitors), p-chloro mercuric benzoate (p-CMB) and β-mercaptoethanol (cysteine inhibitors), iodoacetate and a chelator ethylenediaminetetraacetic acid (EDTA) (metallo protease inhibitors) at 5 mM concentration was studied on protease activity. The reaction mixture (test) was prepared by adding 2.0 ml of purified enzyme, 20 μl of inhibitor, 1.98 ml of deionised water and incubated at 45°C for 30 min before the addition of substrate. The reaction was stopped by the addition of 4 ml of 5% TCA. After 60 min the solution was filtered through Whatman No.1 filter paper and the protease activity of filtrate was measured as described in section 3.2.3.3.

3.4.3.5. Determination of effect of metal ions on protease activity

To determine the effect of various metal ions on protease activity different ions such as Ca²⁺, Mg²⁺, Al³⁺, Co²⁺, Cd²⁺, Fe³⁺, Na⁺, Zn²⁺, Hg²⁺, and Cu²⁺, at 2 mM concentration was added to the reaction mixture. The reaction mixture (test) was prepared by adding 0.5 ml Tris-HCl buffer (50 mM, pH 8) and 0.5 ml of metal ion solution to 0.5 ml of purified enzyme. Reaction mixtures were incubated at 45°C for 30 min, before the addition of substrate. The reaction mixture without metal ion will
serve as control. The reaction was stopped by the addition of 4ml of 5% TCA. After 60 min the solution was filtered through Whatman No.1 filter paper and 1ml of filtrate was mixed with 5ml of 0.4M Na$_2$C$_O_3$ followed by the addition of 0.5ml phenol reagent (1:1 dilution with distilled water). The liberation of tyrosine (appearance of blue colour) was determined at 660nm in a spectrophotometer (ELICO, model SL-164). Relative protease activities were measured as described in the section 3.4.3.2.

3.4.3.6 Determination of effect of different protein substrates on protease activity

To determine the substrate specificity of purified enzyme of *B. subtilis* K-30, hydrolysis of natural protein substrates (BSA, casein, egg albumin, gelatin, collagen and keratin) along with modified substrate (Azocasein) was performed. The reaction mixture was prepared by adding 0.5ml Tris-HCl buffer (50mM, pH 8) and 1.0ml of substrate to 0.5ml of purified enzyme. Reaction mixtures were incubated at 45°C for 30 min. The specific protease activity towards casein was taken as a control.

The reaction was stopped by the addition of 4ml of 5% TCA. After 60 min the solution was filtered through Whatman No.1 filter paper and 1ml of filtrate was mixed with 5ml of 0.4M Na$_2$C$_O_3$ followed by the addition of 0.5ml phenol reagent (1:1 dilution with distilled water). The liberation of tyrosine (appearance of blue colour) was determined at 660nm in spectrophotometer (ELICO, model SL-164). Relative protease activities were calculated as described in section 3.4.3.2.

3.4.3.7 Determination of effect of commercial detergents on protease activity

The compatibility of protease with local laundry detergents was studied in the presence of 10mM CaCl$_2$ and 1M glycine. The detergents used were such as Wheel, Nirma, Surf, Ariel, Henko, Rin and Vim (Ultra). The detergents were diluted in
distilled water (0.7% w/v) and the reaction mixture was prepared by mixing 0.5ml Tris-HCl buffer (50mM, pH 8), 1.0ml of substrate, 1.0ml detergent solution to 0.5ml of purified enzyme. Reaction mixtures were incubated at 45°C to different time intervals (10, 30, 50 and 60 min) and the residual activity was determined. The enzyme activity of control sample (without any detergent) was taken as 100%.

3.5 Production of Alkaline protease with immobilized cells in various matrices by entrapment techniques

3.5.1 Preparation of inoculum

Five milliliters of sterile distilled water was added to a 24 h old slant of *B. subtilis* K-30. The cells were scrapped from the slant into sterile distilled water and the resulted cell suspension at 10% level was transferred aseptically into 250ml Erlenmeyer flasks containing 45ml of sterile inoculum medium. The composition of the inoculum medium is (g/L): glucose, 2.0; casein, 0.5; peptone, 0.5; yeast extract, 0.5, and salt solution, 50ml (salt solution containing [g/L]: KH₂PO₄, 5.0; MgSO₄·7H₂O, 5.0, and FeSO₄·7H₂O, 0.1] and the pH was maintained at 7. The flask was kept in incubator shaker at 150 rpm at 37°C. The content of the flask was centrifuged at 3000 rpm for 10 min and the supernatant was decanted. The cell pellet was washed thoroughly with sterile 20.0g/L potassium chloride solution followed by sodium chloride solution (20.0g/L) and sterile distilled water. Finally the cell mass was suspended in sterile sodium chloride solution (9.0g/L). This cell suspension was used as inoculum for immobilization studies.

3.5.2 Whole cell immobilization in Alginate gel

The alginate entrapment of cells was performed according to the method of Johnsen and Flink (1986). Sodium alginate solution (3%) was prepared by dissolving
sodium alginate in 100ml boiling water and autoclaved at 121°C for 15 min. Both alginate slurry and cell suspension (equivalent to 0.03g dry cell weight, DCW) were mixed and stirred for 10 min to get uniform mixture. The slurry was taken into a sterile syringe and added drop wise into 0.2M CaCl₂ solution from 5cm height and kept for curing at 4°C for 1 h. The cured beads were washed with sterile distilled water 3 to 4 times. When the beads were not used, they were preserved in 0.9% sodium chloride solution in the refrigerator. All operations were carried out aseptically under laminar airflow unit.

3.5.3. Whole cell immobilization in polyacrylamide gel

Immobilization of cells was performed by the method described by Adinarayana and Ellaiah (2005). A cell suspension was prepared by adding 0.03g cells to 10ml chilled sterile distilled water. To another 10 ml of 0.2M sterile phosphate buffer (pH7.0), the following chemicals were added: 2.85g acrylamide (Sigma, USA) 0.15g bisacrylamide, 10mg ammonium per sulphate and 1 ml TEMED. The cell suspension and the above phosphate buffer mixture was mixed well and poured into sterile flat bottom 10cm-diameter petriplates. After polymerization, the acrylamide gel was cut into equal size cubes (4 mm³), transferred to 0.2M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 h for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile distilled water at 4°C until used.

3.5.4 Immobilization of whole cells in Agar-agar

Whole cell immobilization of *B.subtilis* K-30 was carried out in accordance with the method described by Veelken and Pape (1982). A 2% gel was prepared by dissolving 0.4g of agar-agar in 20ml 0f 0.9% sodium chloride solution and sterilized
by autoclaving. The cell suspension (2ml equivalent to 0.03g DCW was added to the molten agar-agar maintained at 40°C, shaken well for few seconds (without forming foam) and poured into sterile flat bottom 4-inch diameter petri plates and allowed to solidify. The solidified agar block was cut into equal sized cubes (4mm$^3$), added to sterile 0.1M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 h for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water 3 to 4 times.

3.5.5 Immobilization of whole cells in gelatin

Five milliliters (0.06% DCW) of cell suspension was added to 15ml of 20% sterile gelatin, maintained at 45°C, and poured into a sterile petri plates. The gel was over layered with 10ml of 5% glutaraldehyde for hardening at 30°C. The resulting block was cut into small sized cubes (4 mm$^3$) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde as described by Veelken and Pape (1982).

3.5.6 Production of Alkaline protease by Batch process with immobilized cells

The immobilized beads (cells equivalent to 0.03g DCW) were transferred to 50ml of production medium in 250ml Erlenmeyer flasks. The composition of production medium was (g/L): glucose, 5; peptone, 7.5, and salt solution, 5% (MgSO$_4$7H$_2$O, 5 g/L; KH$_2$PO$_4$, 5 g/L; and FeSO$_4$.7H$_2$O, 0.1 g/L) with a pH of 9.0. The flasks were incubated at 37°C for 48 h. Samples were withdrawn at regular intervals of 6 h and assayed for alkaline protease activity. After attaining the maximum production of alkaline protease (24 h), the spent medium was replaced with fresh production medium (50ml) and the process was repeated for several batches.
until the beads/blocks started disintegrating. The enzyme titer of each cycle was determined.

3.5.6.1 Alkaline protease assay

The culture broth was centrifuged at 3000 rpm for 20 min and the supernatant served as crude enzyme source. Protease activity was assessed by the modified procedure based on the method of Tsujida et al. (1976) using 1.0% casein in 0.2 M carbonate buffer (pH 10.0) as substrate. One unit of enzyme activity is defined as the amount of enzyme that released 1 μmol of tyrosine/ml/min.